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including conversion of heme a_3 from high spin to low spin and/or disruption of the H-bonding environments surrounding the formyl groups of both heme *a* and heme a_3. To obtain a semi-quantitative measure of the conformational changes induced by the mutations, we use CO as a structural probe. In CcO, the Fe-CO moiety typically exhibits two conformations, called the α and β forms. The Fe-CO stretching mode of the α form is present at ~ 520 cm^{-1}, whereas that of the β forms appears at ~490-495 cm^{-1}. The α form, which is the active conformation of the enzyme, has Fe-CO and C-O stretching modes that do not fall on the $\nu_{Fe\text{-}CO}$*vs* $\nu_{C\text{-}O}$ inverse correlation line characteristic of heme coordinated by a histidine ligand, presumably owing to the interaction of the CO with the nearby Cu_B atom in the binuclear center. Our data of the CO-bound pdC*c*O showed that α/(α+β) intensity ratio varies from nearly zero to one in the mutants. The changes in the α/(α+β) ratio correlate well with changes in some of the heme modes. We postulate that the conformation of the catalytic site, consisting of the two heme groups and Cu_B, is perturbed by the mutations, as indicated by the changes in the heme modes, which disrupts of the juxtaposition between Cu_B and the iron atom of heme a_3 as reflected by the changes in the α/(α+β) ratio. The implications of these results in relation to the measured functional properties of the enzyme will be discussed.

3333-Pos

Characterization of the Radical Intermediates of Dehaloperoxidase A and B from Amphitrite Ornata

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The enzyme intermediates of dehaloperoxidase (DHP) from the marine worm *Amphitrite ornata* are unique within both the globin and cytochrome c peroxidase superfamilies. Both isoenzymes of DHP, termed A and B, have been shown to oxidize trihalophenols to dihaloquinones in a dehalogenation reaction that utilizes hydrogen peroxide as a co-substrate. We have shown that the initially formed heme intermediate in this reaction is not Compound I as is often the case in peroxidases, but rather is a combination of an iron(IV)-oxo (Compound II) and a tyrosyl radical that together have similarity to the Compound ES intermediate of cytochrome c peroxidase. In order to possibly identify the origin of this radical species in DHP, we have expressed the tyrosine mutants DHP A (Y34F), DHP A (Y38F), DHP A (Y34F/Y38F), and DHP B (Y38F), and studied their reaction with hydrogen peroxide using a combination of stopped-flow UV-visible and rapid-freeze quench electron paramagnetic resonance spectroscopies. Although each mutant exhibited an average signal at $g \approx 2.0058$ confirming the presence of a protein radical, significant differences in the lineshape and width of each radical was observed. We have further characterized these mutants using biochemical assays to determine their effect on the catalytic activity of the enzyme, and relate these results to the structure of the heme active site. Such mutagenesis studies of DHP provide critical insight into the mechanistic details of the H_2O_2-dependent oxidative dehalogenation reaction catalyzed by dehaloperoxidase, present a clearer description of the function of DHP at the molecular level, and lead to a better understanding of the paradigms of globin structure-function relationships.

3334-Pos

Investigation of the Low Frequency Dynamics of Heme Proteins: Native and Mutant Cytochromes $P450_{cam}$ and Redox Partner Complexes

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Vibrational coherence spectroscopy investigates the low frequency dynamics of cytochrome $P450_{cam}$ upon binding to its electron transfer partner putidaredoxin (Pd) and its camphor substrate. A strong correlation between the ''detuned'' coherence spectrum and the Raman spectrum is demonstrated. There is a striking appearance of a mode near 103 cm^{-1} in $P450_{cam}$ when camphor is not present in the distal pocket. This reflects a specific heme distortion, such as saddling, in the substrate free state where water is coordinated to the low-spin iron atom. A mode near 78 cm^{-1} intensifies when the $P450_{cam}$/Pd complex is formed, suggesting a ruffling distortion, possibly related to increased electron donation from the thiolate sulfur. The L358P mutant exhibits similar spectroscopic properties to that of wild type $P450_{cam}$ when bound to Pd[1]. The appearance of a mode near 65 cm^{-1} in the coherence spectra of the L358P mutant reveals similarities to the perturbations seen in the $P450_{cam}$/Pd complex, consistent with the view that the heme and its environment in the L358P mutant are similar to the Pd-bound native protein. Resonance Raman spectra are presented for both $P450_{cam}$ and the L358P mutant. When the native and mutant samples are compared, a ~ 5cm^{-1} red-shift of the mode at ~345 cm^{-1} (without substrate) and a ~2cm^{-1} shift (with substrate) are observed. Such changes are due to the thiolate ligand being pushed towards the heme in the mutant sample. Vibrational coherence spectra of the 2Fe-2S cluster proteins ferredoxin (Fd) and putidaredoxin are also presented. Fd displays vibrational overtones of a mode at 44 cm^{-1} mode, along with a weak mode at 283cm^{-1} that is associated with the labile sulfur-iron stretching vibration.

[1] T.Tosha *et al*., *J.Biol.Chem*., **2004**, 279, 42836.

3335-Pos

Block the Inhibitor Binding Site in the Interior of Dehaloperoxidase from Amphitrite Ornata

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Dehaloperoxidase (DHP A) from the annelid *Amphitrite ornata* is a catalytically active hemoglobin-peroxidase that possesses an internal binding site in the distal pocket and an external binding site near the heme edge. We have recently demonstrated that DHP A has a unique two-site competitive binding mechanism, in which the internal and external binding sites communicate through two conformations of the distal histidine (H55). The native substrate is 2,4,6-tribromophenol, but DHP A is capable of oxidizing any 2,4,6-trihalophenol to the corresponding dihaloquinone and other products. While DHP A is very effective at oxidizing 2,4,6-trichlorophenol, assays of DHP A on 2,4-dichlorophenol and 4-chlorophenol show little to no activity. Binding of 4-halophenols in the internal site prevents oxidation of trihalogenated phenols at the external site, i.e. they are inhibitors. X-ray crystallography shows that when para-halogenated phenols (4-iodo-, 4-bromo-, 4-chlorophenol) bind internally, the halogen is accommodated by a hydrophobic cavity that is analogous to the Xenon 4 binding site in Sperm whale myoglobin. Using resonance Raman spectroscopy, we demonstrate that the apparent dissociation constants of the para-halogenated phenols mimic the trend observed in the X-ray crystal structures. The results suggest that a few amino acids (L100, V59, F21, F24, and F35) surrounding the hydrophobic cavity regulate internal binding of the inhibitor. Using site-directed mutagenesis, we have changed several of these amino acids to prevent internal binding, and thus to increase DHP A activity towards mono- and dichlorinated phenols. Mutation to tryptophan (F21W and V59W) gives rise to new radical intermediates which complicates the interpretation in terms of inhibitor binding. Alternatively, aliphatic amino acids and phenylalanine provide steric effects that can alter the oxidation of rates of 4-chloro-, and 2,4-dichlorophenol.

3336-Pos

Cytochrome c Oxidase CuA and Heme A: Redox Equilibrium and Interactions

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Reduction of detergent-solubilized formate-inhibited beef heart cytochrome c oxidase +/- cytochrome c in turnover with ascorbate was followed aerobically. Heme c, heme a and CuA steady states were monitored. Heme a and CuA were in equilibrium with each other, and with cytochrome c when the latter was present. In the formate system there is no aerobic reduction of any binuclear centre component (heme a3 or CuB). At pH 7.4 and 30 deg C calculated E0'values were +310 mV for heme a and +260 mV for CuA, assuming E0'for cyt. c of +255 mV. The difference in heme a and CuA redox potentials permits determination of separate difference spectra for the two. Oxidized (cupric) beef heart CuA has positive absorbancies in the 500-600 nm region in addition to the characteristic 835 nm band, as previously shown for some isolated bacterial oxidase CuA-containing subunit II preparations. The 605nm peak of reduced heme a is red-shifted in presence of oxidized CuA. Both heme a and CuA titrations are close to simple Nernstian one-electron processes, indicating almost no redox interaction between the centres in the formate-inhibited system. Reported difference spectra of bacterial cytochrome c oxidases and separated CuA-containing subunits show similar features in the visible region but marked differences in the NIR bands, with beef heart and Rhodobacter CuA red-shifted compared with Paracoccus and Thermus CuA. The results affect the kinetic analysis of the enzyme as well as the use of the CuA steady state in determining the functional status of the terminal oxidase in vivo.

3337-Pos

Thermodynamic Profiles of Heterotropic Allostery of Hemoglobin (Hb) by Isothermal Titration Calorimetry (ITC)

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The O_2-affinities of deoxy- and oxy-states of Hb ($K_T$and K_R) are reduced up to 60- and 2,000-folds, respectively, by interactions with potent heterotropic allosteric effectors such as BPG, IHP, BZF, and L35 (1,2). These heterotropic allosteries of such magnitudes mean that heterotropic allosteries are the principal regulatory function of Hb rather than the homotropic allostery (cooperativity), since the latter provides an allostery of only ~30-folds (1). It should be noted